

DESCRIPTION

CULTURED *XENOPUS LAEVIS* CELL LINES EXPRESSING MUTANT ADENOMATOUS
POLYPOSIS COLI GENE

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Technical Field

The present invention relates to mutant APC proteins that can induce piling up of cells, and to cells expressing these proteins.

10 Background Art

The *adenomatous polyposis coli* (APC) gene is a tumor suppressor gene identified as a causative gene of Familial Adenomatous Polyposis (FAP). It has been shown that mutation of the APC gene is not restricted to FAP, but is involved also in the development of
15 non-hereditary adenomatous polyposis and colon cancer (Polakis, P., Biochim Biophys Acta. (1997) 1332, F127-147). In FAP patients, many benign polyps form in the colon, from which malignantly transformed cancer cells develop. Although colon cancer develops through multistep changes that occur in a great number of oncogenes and tumor
20 suppressor genes, mutations in the APC gene are found at the earliest stage. Therefore, APC gene abnormalities are considered to be causes of colon cancer.

The APC protein (hereinafter, APC) is a large protein having a molecular weight of approximately 310 kDa comprising 2843 amino
25 acids, and binds to a great number of proteins. To date, it has been reported to bind directly to the B56 subunit of PP2A phosphatase; APC-stimulated guanine nucleotide exchange factor (Asef); KAP3/KIF3A/KIF3B microtubule-associated motor protein complex belonging to the kinesin family; β -catenin/GSK-3 β /Axin, a constituent
30 of the Wnt signal transduction pathway; microtubules, which are cytoskeleton components; proteins of the microtubule-associated protein EB/RP family; p34^{cdc2} kinase, a cell cycle regulatory factor; Siah-1, an apoptosis-related protein; and hDLG, a tumor suppressor gene product (for a review, see Bienz, M., Nat. Rev. Mol. Cell Biol.
35 (2002) 3(5), 328-338). In the polyps and colon cancer cells of FAP patients, mutations occur in both of the APC alleles, and only mutant

APCs that have lost their C-terminal regions due to a truncation in the middle are expressed. Cells are thought to become cancerous because such mutant APCs exhibit abnormal functions.

APC has been reported to promote β -catenin degradation by complexing with β -catenin/GSK-3 β /Axin, and to function in maintaining a low level of existing β -catenin (Rubinfeld, B., Science (1993) 262:1731-1734; Su, L. K., Science (1993) 262:1734-1737). When β -catenin is present in large amounts, it is transferred into the nucleus where it binds to a transcription factor and promotes cell proliferation. Therefore, initially, APC was considered to function as a tumor suppressor protein by regulating the level of existing β -catenin. However, detailed observation of the polyp-forming process using FAP model mice showed that epithelial cells which had differentiated in the crypt region could not migrate normally to the villus apex, and therefore dropped into the inside of the villus at the crypt-villus boundary, becoming morphologically different from their original form, resulting in polyps (Oshima, M., et al., Cancer Res. (1997) 57 (9), 1644-1649). Furthermore, promotion of cell proliferation does not take place inside the polyps, and epithelial cells that develop morphologies different from the original state while maintaining an intercellular adhesive structure were proved to be the cause of polyp formation. Therefore, APC is now considered to be involved in regulating cell morphology and motility. Furthermore, APC was found to bind to Asef, which functions in the regulation of the actin cytoskeleton (Kawaki, Y., et. al. Science (2000) 289(5482), 1194-1197), and to microtubules at the leading edges of motile cells (Mimori-Kiyosue, Y. et. al., J. Cell Biol. (2000) 148(3), 505-518). The possibility of APC involvement in cytoskeleton regulation is beginning to be considered.

Elucidation of the functions of normal and mutant APCs in cell morphology and motility, and elucidation of the causes of polyp formation and malignant transformation have long been anticipated.

Disclosure of the Invention

The present invention was achieved in view of the above circumstances. An objective of the present invention is to elucidate

the functions of normal and mutant APCs in cell morphology and motility. Another objective of the present invention is to provide uses of the mutant APC proteins based on the obtained findings.

To elucidate APC functions in detail, the present inventors
5 expressed full-length and truncated APCs labeled with an
autofluorescent protein (GFP; green fluorescent protein) in
epithelial cells. When the fusion protein cDNAs were introduced into
cells, and several strains from each independent clone stably
expressing the fusion proteins were isolated by drug resistance,
10 protruding cell layers were observed as a common feature among each
of the clones that express the truncated structurally-different APCs.
Observation under electron microscope showed that intercellular
adhesive structures (AJ, TJ) are formed, but cell adhesion attenuates
due to increased intercellular space and cell-substrate space, and
15 the cells are piled up. In cell lines expressing full-length APC,
piling up of cells did not occur and cell adhesion was strengthened.
Maintenance of the intercellular adhesive structures in the cluster
of piled up cells was confirmed by immunohistostaining. These
results showed that mutant APCs cause piling up of cells, leading
20 to the completion of this invention. The present inventors
discovered, for the first time, the phenomenon that mutant
APC-expressing cells cause piling up of cells while maintaining
intercellular adhesive structures.

The mutant APCs of this invention inhibit the monolayering of
25 normal epithelial cells, and cause the cells to pile up while
maintaining cell-cell adhesion. Therefore, cells that express the
mutant APCs of this invention are useful as model cells for the
polyp-forming phenomenon. Furthermore, the mutant APC-expressing
cells of this invention can be used to screen compounds that inhibit
30 piling up of cells.

That is, the present invention relates to mutant APC proteins
that may induce piling up of cells, cells that express these proteins,
and uses of these cells. More specifically, the present invention
relates to:

35 (1) a mutant APC protein comprising the function of inducing
piling up of cells;

(2) the mutant APC protein of (1), wherein at least any one of the amino acid regions of (a) to (c) is deleted:

(a) C-terminal amino acid region starting from amino acid position 2827 in the APC protein of SEQ ID NO: 1;

5 (b) C-terminal amino acid region starting from amino acid position 2159 in the APC protein of SEQ ID NO: 1; and

(c) C-terminal amino acid region starting from amino acid position 860 in the APC protein of SEQ ID NO: 1;

10 (3) the mutant APC protein of (1) or (2), wherein the protein is derived from *Xenopus laevis*;

(4) a mutant APC protein which can induce piling up of cells, wherein said protein comprises the amino acid sequence of a mutant APC protein of any one of (1) to (3), in which one or more amino acids are substituted, deleted, added, and/or inserted;

15 (5) a polynucleotide that encodes a mutant APC protein of any one of (1) to (4);

(6) a vector that comprises the polynucleotide of (5);

(7) a cell that comprises an artificially expressed mutant APC protein of any one of (1) to (4), or the vector of (6);

20 (8) the cell of (7), which is derived from a mammal;

(9) the cell of (7), which is derived from *Xenopus laevis*;

(10) a cell of any one of (7) to (9), which is an established cell line;

25 (11) a method of screening for a candidate compound that inhibits piling up of cells, wherein the method comprises the steps of:

(a) contacting a cell of any one of (7) to (9) with a test compound;

(b) detecting piling up of said cells; and

30 (c) selecting compounds that inhibit the piling up of cells; and,

(12) a method of screening for a polynucleotide that encodes a mutant APC protein which can induce piling up of cells, wherein the method comprises the steps of:

35 (a) introducing *Xenopus laevis*-derived cells with a test polynucleotide to express a mutant APC protein;

- (b) culturing said cells;
- (c) detecting piling up of said cells; and
- (d) selecting polynucleotides that pile up said cells.

5 The present invention provides mutant APC proteins (which are abbreviated as "mutant APCs" in parts of this specification). Expression of the mutant APCs of this invention in cells induces piling up of cells. The mutant APCs of this invention were identified by the present inventors as proteins that induce piling up of cells while
10 maintaining cell-cell adhesion.

 In the present invention, the term "mutant" refers to a protein comprising the amino acid sequence of a normal APC protein, in which one or more amino acids have been substituted, deleted, added, or inserted. Furthermore, the "mutation" may be a conservative mutation
15 in which the amino acid used for the substitution has structural or chemical characteristics similar to those of the substituted amino acid, or a mutation involving non-conservative substitution. That is, the types of mutations present in the mutant APCs of this invention are not particularly limited, as long as they have the function of
20 inducing piling up of cells. The mutant APCs of the present invention are usually produced artificially (or by modifying normal APCs), or obtained by isolation and purification.

 APC proteins have so far been discovered in a variety of organisms. The amino acid sequences of these normal APC proteins can
25 be obtained easily from public databases. For example, the GenBank accession numbers for the amino acid sequences of human APC protein and *Xenopus laevis* APC protein are M74088 and U64442, respectively. The amino acid sequence of *Xenopus laevis* APC protein is shown in SEQ ID NO: 1.

30 The mutant APCs of this invention are not limited to specific types of APC mutants. An example of the mutant APCs of this invention is a mutant of the *Xenopus laevis* APC protein comprising the amino acid sequence of SEQ ID NO: 1.

 In a preferred embodiment of the present invention, the mutant
35 APCs are proteins in which a portion of the amino acid region of a normal APC has been deleted (truncated APC proteins).

The mutant APCs of this invention are normally proteins in which the three amino acids of a TSV sequence (DLG-binding region) at the C-terminus are deleted. This TSV sequence is recognized as a PDZ protein binding motif, and binding of the hDLG protein, which is one of the PDZ proteins, is reported to occur in this region (Matsumine, A., et al., Science (1996);272(5264):1020-3). Furthermore, the mutant APCs of this invention preferably comprise an N-terminal amino acid region containing at least a "heptad repeat" and "armadillo repeat", but the mutant APCs of this invention are not limited to those containing these repeats.

More specifically, examples of the mutant APCs of this invention are proteins having a structure in which at least one of the following amino acid regions of (a) to (c) is deleted:

- (a) C-terminal TSV sequence (DLG-binding region), or more specifically, the C-terminal amino acid region starting from amino acid position 2827 in the APC protein of SEQ ID NO: 1;
- (b) C-terminal basic region and TSV sequence, or more specifically, the C-terminal amino acid region starting from amino acid position 2159 in the APC protein of SEQ ID NO: 1; and
- (c) a 15 amino-acid repeat, 20 amino-acid repeat, and C-terminal basic region and TSV sequence, or more specifically, the C-terminal amino acid region starting from amino acid position 860 in the APC protein of SEQ ID NO: 1.

The present invention also comprises proteins that have the function of inducing piling up of cells, and comprise the amino acid sequences of the above-mentioned proteins, in which one or more amino acids are deleted, substituted, or added. Methods for preparing these proteins include methods that use hybridization techniques or gene amplification techniques. More specifically, one skilled in the art can use hybridization techniques (Current Protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 6.3-6.4) to isolate DNAs that are highly homologous to mutant APCs, based on the DNA sequences encoding the above mutant APCs, or a portion thereof, from DNA samples derived from an organism of the same or different species, and consequently obtain the proteins of this invention. The proteins of the present invention also comprise

proteins that are encoded by DNAs hybridizing with the DNAs encoding the thus isolated proteins, and which have the function of inducing piling up of cells.

Stringent hybridization conditions of "1x SSC, 0.1% SDS, 37°C" are normally used for isolating a DNA encoding a protein that comprises an amino acid sequence of mutant a APC in which one or more amino acids are deleted, substituted, or added. More stringent conditions are "0.5x SSC, 0.1% SDS, 42°C", and even more stringent conditions are "0.2x SSC, 0.1% SDS, 65°C". As the hybridization conditions become more stringent, isolation of DNAs having high homology to the probe sequence can be expected. However, the above combinations of SSC, SDS, and temperature conditions are only examples. Those skilled in the art can achieve similar stringencies as those described above by appropriately combining the above-mentioned factors or other factors that determine the stringency of hybridization (for example, probe concentration, probe length, and hybridization reaction time).

Polypeptides encoded by DNAs that are isolated using such hybridization techniques are usually highly homologous to the mutant APCs of this invention at the amino acid sequence level. Highly homologous refers to a sequence homology of at least 40% or higher, preferably 60% or higher, more preferably 80% or higher, even more preferably 90% or higher, still more preferably 95% or higher, and yet more preferably 97% or higher (for example, 98-99%).

The degree of homology of one amino acid sequence to another can be determined by following, for example, the BLAST algorithm by Karlin and Altschl (Proc. Natl. Acad. Sci. USA, 87: 2264-2268, 1990; Proc. Natl. Acad. Sci. USA, 90: 5873-5877, 1993). Programs such as BLASTX were developed based on this algorithm (Altschul et al. J. Mol. Biol. 215: 403-410, 1990). In the analysis of amino acid sequences using BLASTX, the parameters are, for example, score= 50 and word length= 3. When using the BLAST and Gapped BLAST programs, the default parameters for each program are used. The specific techniques of these analyses are known in the art (<http://www.ncbi.nlm.nih.gov>).

In the present invention, the phrase "piling up of cells" can be described, for example, by the cellular conditions (forms)

described below, but are not particularly limited to these conditions since an exact definition is usually difficult.

(a) A height-wise overlapping of multiple nuclei from different cells is observed.

5 (b) Cell bodies do not dissociate from the cell layer, and exist at a position that is clearly higher than the average height (2-4 μm) of monolayered cells (for example, A6 cells).

10 (c) regions where cell bodies and cell nuclei are observed at the electron microscope level to be present in multiples and in a height-wise manner, are observed to have closely-winding "wrinkles" in the cellular monolayer at the light microscope level (phase contrast microscopy).

In the present invention, one skilled in the art can determine whether a protein can induce piling up of cells using known methods.
15 For example, whether cells expressing a target protein are piled up can be confirmed readily by phase contrast microscopy or fluorescent antibody methods.

Those skilled in the art can produce mutant APCs of this invention by commonly known methods (PCR and such), for example, by
20 using the nucleotide sequence of a DNA encoding a normal APC protein as a template. For example, the above mutant APC proteins can be produced by PCR amplification of the DNAs that encode these proteins, using the cDNA or genomic DNA of normal APCs as templates, and by cloning these DNAs into expression vectors. The cDNAs of normal APCs
25 can be obtained by those skilled in the art by known methods, for example, by constructing a cDNA library from cells expressing normal APCs, and then performing a hybridization using a portion of the APC cDNA as a probe. The cDNA library may be prepared, for example, by a method described in literature (Sambrook, J. et al., Molecular
30 Cloning, Cold Spring Harbor Laboratory Press (1989)), or a commercially available DNA library may also be used.

More specifically, the above mutant APCs may be produced by methods presented in the Examples below. Those skilled in the art can appropriately modify methods shown in the Examples below to
35 produce mutant APCs to carry any amino acid region of a normal APC.

Furthermore, a mutant APC of this invention can be produced by

modifying the amino acid sequence of a normal APC protein (for example, the amino acid sequence of SEQ ID NO: 1). For example, an amino acid sequence can be modified by modifying the nucleotide sequence of a DNA encoding a normal APC protein, using well-known methods such as site-directed mutagenesis (Current Protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 8.1-8.5), and expressing the DNA with a modified nucleotide sequence in an appropriate cell. Since amino acid mutations in proteins may occur in a natural environment (in nature), the proteins of this invention include isolated natural mutant APC proteins as long as they comprise the function of inducing piling up of cells while maintaining cell-cell adhesion, although the mutant APCs of this invention are normally produced artificially.

The mutant APCs of the present invention can be prepared as recombinant polypeptides by methods well known to those skilled in the art. A recombinant polypeptide can be prepared, for example, by inserting a DNA encoding a mutant APC of this invention into an appropriate expression vector, collecting a transformant obtained by transfecting the vector into an appropriate host cell, and purifying the polypeptide after obtaining an extract by chromatography such as ion exchange chromatography, reverse phase chromatography, gel filtration, chromatography, or affinity chromatography in which an antibody against the mutant of this invention is fixed onto a column, or by a combination of these columns.

Furthermore, when a mutant APC of the present invention is expressed in a host cell (for example, an animal cell or *E. coli*) as a fusion polypeptide with a glutathione S-transferase protein or recombinant polypeptide with multiple histidine residues, the expressed recombinant polypeptide can be purified using a glutathione column or a nickel column. After the fused polypeptide is purified, regions other than the desired mutant can be removed from the fused polypeptide, as necessary, by cleavage with thrombin, factor Xa, etc.

The present invention also includes polynucleotides encoding the mutant APCs of this invention. The polynucleotides comprise DNAs that encode the mutant APCs of this invention, and RNAs which are transcription products of these DNAs.

Furthermore, the present invention provides vectors into which DNAs of the present invention are inserted. The vectors of the present invention are useful in expressing or producing the mutant APCs of the present invention in cells.

5 When *E. coli* is used as a host cell, there is no particular limitation other than that the above-mentioned vector should have an "ori" and a marker gene. The "ori" is used to amplify and mass-produce the vector in *E. coli* (e.g., JM109, DH5 α , HB101, or XL1Blue and such). The marker gene is used to select the transformed
10 *E. coli* (e.g., a drug-resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, or chloramphenicol). For example, M13-series vectors, pUC-series vectors, pBR322, pBluescript, pCR-Script, and such can be used. In addition to the above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used
15 for the subcloning and excision of cDNAs. When an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order to be amplified in *E. coli*. Additionally, when *E. coli* such as JM109, DH5 α , HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ
20 promoter (Ward et al. (1989) Nature 341:544-546; (1992) FASEB J. 6:2422-2427), araB promoter (Better et al. (1988) Science 240:1041-1043), or T7 promoter, that allows efficient expression of the desired gene in *E. coli*. Other examples of the vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET
25 (where BL21, a strain expressing T7 RNA polymerase, is preferably used as the host).

Furthermore, the vector may comprise a signal sequence for polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the pelB signal sequence (Lei, S. P. et al.
30 (1987) J. Bacteriol. 169:4379) may be used as a signal sequence for polypeptide secretion. For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a host cell.

In addition to *E. coli*, expression vectors derived from mammals
35 (e.g., pCDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18(17):5322), pEF, pCDM8), insect cells (e.g., "Bac-to-BAC

baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g., pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also
5 be employed to express the mutant APCs of the present invention.

In order to express proteins in animal cells such as CHO, COS, and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan et al. (1979) *Nature* 277:108), MMLV-LTR promoter, EF1 α promoter
10 (Mizushima et al. (1990) *Nucleic Acids Res.* 18:5322), CMV promoter, etc.). It is even more preferable that the vector also carries a marker gene for selecting transformants (for example, a drug-resistance gene selected by a drug such as neomycin and G418. Examples of vectors with such characteristics include pMAM, pDR2,
15 pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

Examples of methods for expressing the mutant APCs of the present invention in animals include inserting a DNA of this invention into an appropriate vector, and introducing the vector into a living body by retrovirus methods, liposome methods, cationic liposome
20 methods, adenovirus methods, and such. The vectors used in these methods include, but are not limited to, adenovirus vectors (e.g., pAdexlcw), retrovirus vectors (e.g., pZIPneo), and such. General gene manipulation techniques, such as inserting a DNA of the invention into a vector, can be performed by conventional methods (*Molecular Cloning*, 5.61-5.63). Administration to a living body may be
25 performed by *ex vivo* or *in vivo* methods.

The present invention also provides cells transfected with the vectors of this invention. In a preferred embodiment of the present
30 invention, the cells are those that artificially express the mutant APCs of this invention (for example, cells in which mutant APCs are artificially introduced and expressed). There are no particular limitations as to the kinds of cells into which the vectors of the present invention are introduced, and for example, *E. coli* and various
35 animal cells may be used. In a preferred embodiment of the present invention, the cells are epithelial cells having the characteristic

of piling up while maintaining cell-cell adhesion due to expression of the mutant APCs of this invention. The cells of the present invention are more preferably established cell lines that stably express the mutant APCs of this invention. Furthermore, the cells of the present invention are preferably cells derived from animals, and more preferably cells derived from *Xenopus laevis* (for example, A6 cells).

Cells that express the mutant APCs of this invention are considered to be similar to the actual cells that form polyps due to APC mutation. In Familial Adenomatous Polyposis (FAP) patients, normal cell-cell adhesion occurs at an early stage, and then benign polyps are formed, which are non-infiltrative and have yet to become malignant. Normally, several hundreds to several thousands of such polyps are formed, and if they are left alone and not excised, malignant cells develop from them due to mutations also in genes besides APC. At this point, canceration is considered to occur. Although cells expressing the mutant APCs of this invention represent a condition that mimics only APC mutations, these cells are useful as research materials for elucidating the causes of polyp formation or canceration.

Furthermore, the cells of the present invention are useful for screening compounds that inhibit piling up of cells. When the cells expressing the mutant APCs of this invention are *Xenopus laevis*-derived cells, the advantage is that the cells can be cultured at room temperature, in a carbon dioxide-free environment.

The cells of the present invention can be used as a production system to produce and express the mutant APCs of the present invention. The systems for producing mutant APCs include *in vitro* and *in vivo* systems. Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

Eukaryotic host cells that can be used are, for example, animal cells, plant cells, and fungi cells. Mammalian cells, for example, CHO (J.Exp.Med. (1995) 108:945), COS, 3T3, myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells (e.g., *Xenopus* cells), or insect cells (e.g., Sf9, Sf21, Tn5) are known as animal cells. Among CHO cells, those defective in the DHFR gene, dhfr-CHO (Proc. Natl. Acad.

Sci. USA (1980) 77:4216-4220), and CHO K-1 (Proc. Natl. Acad. Sci. USA (1968) 60:1275) are particularly preferable. Among animal cells, CHO cells are particularly preferable for large-scale expression. A vector can be introduced into a host cell by, for example, calcium phosphate methods, DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, and lipofection methods.

Useful prokaryotic cells comprise bacterial cells. As examples of bacterial cells, *E. coli* (for example, JM109, DH5 α , HB101, and such), and also *Bacillus subtilis* are known.

These cells are transformed by a desired DNA, and the transformants are cultured *in vitro* to obtain a polypeptide. Transformants can be cultured using known methods. For example, the culture medium for animal cells may be a culture medium such as DMEM, MEM, RPMI1640, or IMDM, and may be used with or without serum supplements such as fetal calf serum (FCS).

Production systems using animal and plant hosts may be used as systems for producing polypeptides *in vivo*. For example, DNAs that encode the mutant APCs of the present invention can be introduced into those animal or plant hosts; the mutant APCs are produced in the body of the animal or plant and then recovered.

Animals to be used for the production system described above include mammals and insects. Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser (1993) SPECTRUM Biotechnology Applications). Alternatively, the mammals may be transgenic animals.

For instance, DNAs that encode the mutant APCs of the present invention may be prepared as a fusion gene with a gene that encodes a polypeptide specifically produced in milk, such as the goat β casein gene. DNA fragments comprising the fusion gene are injected into goat embryos, which are then introduced back into female goats. The mutant APCs of this invention can be obtained from the milk produced by the transgenic goats (i.e., those born from the goats that received the modified embryos) or from their offspring. Appropriate hormones may be administered to increase the volume of milk containing the polypeptides produced by transgenic goats, (Ebert, K. M. et al.,

(1994) Bio/Technology 12:699-702).

Alternatively, insects such as silkworms may be used. Baculoviruses into which a DNA encoding the mutant APCs of this invention has been inserted, can be used to infect silkworms, and
5 the mutant APCs can be recovered from body fluids (Susumu M. et al., (1985) Nature 315:592-594).

In addition, when using plants, tobacco, for example, can be used. When using tobacco, a DNA encoding a mutant APC of this invention may be inserted into a plant expression vector, such as
10 pMON 530, which is then introduced into bacteria such as *Agrobacterium tumefaciens*. Then, the bacteria are used to infect tobacco such as *Nicotiana tabacum*, and the desired mutant APCs are recovered from the leaves (Julian K.-C. Ma et al., (1994) Eur. J. Immunol. 24:131-138).

15 The mutant APCs of the present invention, obtained as above, may be isolated from the inside or outside (the medium and such) of host cells, and purified as a substantially pure homogeneous polypeptide. Purification methods are not limited to any specific method. In fact, any standard method for isolating and purifying
20 polypeptides may be used. For instance, column chromatography, filters, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric point electrophoresis, dialysis, and recrystallization may be
25 appropriately selected and combined to isolate and purify polypeptides.

Chromatographies such as affinity chromatography, ion-exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and
30 adsorption chromatography may be used (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed. Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press (1996)). These chromatographies may be performed using liquid chromatographies such as HPLC and FPLC. The present invention provides highly purified
35 mutant APCs purified by the above methods.

Generally, a widely-used assay for the Wnt signal transduction system involves the method of injecting a small amount of Wnt signal factor mRNA, antibody, and such into *Xenopus* oocytes, and examining the effects on development (Gloy, J., et al., Nat. Cell Biol. (2002) 4(5):351-357). This technique is also used for APC and for APC-binding proteins, such as β -catenin and axin, and phenomena such as formation of double-headed tadpoles and abnormal head-formation have lead to discussions on the function of APC and APC-binding proteins in the Wnt signal transduction system. Therefore, the purified mutant APCs of the present invention can also be used in this type of experiments.

Furthermore, this invention provides methods of screening for candidate compounds as agents that inhibit piling up of cells. The screening methods of this invention comprise the steps of: (a) contacting cells expressing a mutant APC of this invention with a test compound; (b) detecting piling up in these cells; and (c) selecting a compound that inhibits the piling up of cells.

The test compounds used in the screening methods of this invention are not particularly limited. Examples include libraries of synthetic low-molecular-weight compounds, purified proteins, gene library expression products, synthetic peptide libraries, cell extracts, and cell culture supernatants.

Usually, "contact" in step (a) mentioned above can be carried out by adding a test compound into a culture solution of cells expressing a mutant APC of this invention, but is not particularly limited to this method.

"Detecting piling up of cells" in step (b) mentioned above can be performed by those skilled in the art using the above-mentioned methods (for example, methods that use phase contrast microscopy and such).

In step (c) mentioned above, normally when the degree of piling up of cells is decreased compared to that when the test compound is not contacted with the cells, piling up of cells is considered to be inhibited.

Since piling up of cells is observed in many cancer cells, compounds that inhibit piling up of cells are expected to have

antitumor activity. Therefore, compounds selected by the screening methods of this invention are expected to have antitumor activity. More specifically, agents that inhibit piling up of cells, which comprise these compounds as active ingredients, may become potential antitumor agents.

In conventional *in vitro* cell culture systems, "fibroblast" transformation assays with oncoproteins such as Ras and Src, are widely used. These assays are for observing, using a light microscope, the "focus" formation phenomenon that is caused by an increase of cells that do not adhere to anywhere since they continue to proliferate after the loss of anchorage dependence. In most cases when Ras, Src, or a number of oncogenes are expressed in epithelial cells, cell-cell adhesion and epithelial polarity are known to be lost, such that the cells take on a fibroblast or mesenchymal cell-like form. The cells of the present invention are cells that pile up while maintaining cell-cell adhesion, and thus differ from conventional cells. Therefore, the methods of this invention using these cells enable the screening of compounds that were difficult to obtain by conventional methods (for example, compounds that specifically inhibit the "piling up of epithelial cells").

Despite the fact that many cancers are derived from epithelial cells, the mainly used assay system that employed cultured cells were the "focus" formation assays, which use the above-mentioned fibroblasts. Development of assay systems that use epithelial cells may provide a more effective screening system against many kinds of cancer cells. The assay systems are also useful as materials for elucidating the mechanisms of how epithelial cells maintain monolayers.

The present invention comprises compounds obtained by the above-mentioned screening methods, and agents that inhibit piling up of cells or antitumor agents that contain these compounds as active ingredients.

The above-mentioned pharmaceutical agents of the present invention can be formulated by known pharmaceutical methods. For example, they can be formulated by appropriately combining with pharmaceutically acceptable carriers or vehicles, more specifically,

sterilized water or physiological saline, vegetable oil, emulsifiers, suspending agents, surfactants, stabilizers, flavoring compounds, excipients, vehicles, preservatives, binding agents, and such, and by mixing at a unit dosage and form required by accepted pharmaceutical implementations.

Furthermore, the present invention relates to methods of screening for polynucleotides encoding mutant APCs, which can induce piling up of cells. The screening methods comprise the step of introducing a test polynucleotide into *Xenopus laevis*-derived cells to express mutant APC proteins, culturing these cells, detecting piling up of these cells, and selecting polynucleotides that cause the piling up of these cells.

In a preferred embodiment of the present invention, whether a polynucleotide encoding a subject's APC causes polyp development may be examined by using such a polynucleotide as a test sample. Usually, the test polynucleotide is inserted into an expression vector suitable for cellular expression, and then introduced into cells. Those skilled in the art can appropriately select an expression vector, and use standard genetic engineering techniques to construct an expression vector that carries this polynucleotide. This expression vector can be introduced into cells by known methods, such as the electroporation methods mentioned above, or lipofection methods.

An example of *Xenopus laevis*-derived cells used in the above-mentioned screening methods is A6 cells. Furthermore, piling up of cells can be detected by, for example, a method utilizing a phase contrast microscope. More specifically, a detailed observation of the cell morphology using the methods presented in the following Examples will enable detection of piling up of cells. In the present screening methods, a test polynucleotide is judged to code for a mutant APC that may induce piling up of cells, when piling up is observed in the cells expressed with the polynucleotide.

Brief Description of the Drawings

Fig. 1 shows a block diagram of *Xenopus laevis* APC protein domains, and a schematic diagram of the GFP-fused full-length and mutant APCs used in the present invention.

Fig. 2 shows a set of phase-contrast images of parental A6 cells, and A6 cell lines stably expressing GFP-fAPC, GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP.

Fig. 3 is a set of scanning electron microscope images showing piling up of cells expressing mutant APCs.

Fig. 4 is a set of transmission electron microscope images showing piling up of cells expressing mutant APCs.

Fig. 5 demonstrates that even after piling up of cells expressing mutant APCs, cells form an intercellular adhesive structure. TJ protein ZO-1 was stained with anti-ZO-1 antibody.

Fig. 6 is a set of fluorescence microscope images showing localization of GFP-fAPC, GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP, together with DLG localization, in the absence of microtubules in non-polarized A6 cells. GFP-fAPC co-localizes with DLG in a striated manner (arrows), while GFP-APC(Δ TSV) is dispersed in the cytoplasm (arrow heads) and does not co-localize with DLG.

Fig. 7 is an area plot of cells at a given period of time after parental A6 cells and cells expressing GFP-fAPC, GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP are plated. Assays were performed in the absence of nocodazole, as shown on the left; and under conditions in which the microtubules were depolymerized in the presence of nocodazole, as shown on the right.

Best Mode for Carrying out the Invention

The present invention will be explained in detail below with reference to Examples, but it is not to be construed as being limited thereto. The antibodies and cells used in Examples are as follows. Anti-ZO-1 monoclonal antibody (Clone: T8-754) and anti-DLG antibody (anti-PSD-95 Family, clone K28/86.2), were purchased from Sanko Junyaku and Upstate Biotechnology, respectively. TOTO-3 that selectively stains nucleic acids, and rhodamine-phalloidin that selectively stains actin filaments, were purchased from Molecular Probe. Nocodazole, a pharmaceutical agent that depolymerizes microtubules, was purchased from SIGMA. A6 cells (*Xenopus laevis* renal epithelial cell line) were grown in Leibovitz's L-15 medium containing 10% fetal calf serum (GIBCO BRL) at 23°C in a carbon

dioxide-free environment. The genes and cells of *Xenopus laevis* were chosen because of the reason described in Example 2.

[Example 1] Construction of autofluorescent protein GFP (green fluorescent protein)-fused APCs

In order to elucidate APC function in detail, a routine procedure was used to prepare expression vectors in which cDNAs carrying different APC sites were fused with the cDNA of an autofluorescent protein, GFP (green fluorescent protein) (Fig. 1).

The *Xenopus laevis* APC gene sequence is known and is described in GenBank (U64442). The individual expression vectors were produced by the following procedure.

(a) GFP-fAPC: a vector for expressing GFP-fused full-length APC.

Expression vectors, pGFP-C(NheI)/APC(1-8490) and pQBI25/APC(1-8487), which carry full-length APCs have already been reported in the present inventors' publications. In order to construct a GFP-fAPC expression vector, first, a cDNA containing the 3' region of APC, which had been excised from pGFP-C(NheI)/APC(1-8490) using *Xba*I, was inserted into the *Xba*I site of pEGFP-C1 (Clontech). Then, to the *Sac*II-*Bam*HI site of this vector, cDNA excised from pQBI25/APC(1-8487) using *Sac*II and *Bam*HI was inserted. Finally, cDNA excised from pGFP-C(NheI)/APC(1-8490) using *Bam*HI and *Not*I was inserted into the *Bam*HI-*Not*I site of this vector. A vector containing a full-length APC (APC/pEGFP-C1) was thus constructed.

(b) GFP-APC(Δ TSV): a vector expressing TSV-deleted APC. TSV is three amino acids at the APC C-terminus. A gene encoding amino acids 2089-2826 of APC was produced by PCR using pGFP-C(NheI)/APC(1-8490) as the template and the following primers:

CGACGCGTAATGCATTTTCTCCAGACTCTG (SEQ ID NO: 2), and
GGAATTCGGATCCTCACACCAGATAAGAACCAGAGTGCC (SEQ ID NO: 3).

This PCR product was cleaved with *Spe*I and *Eco*RI, and then inserted into the *Nhe*I-*Eco*RI site of pGFP-C(NheI) vector

(APC(6475-8478)/pGFP-C(*NheI*)). This vector was cleaved with *PvuI* and *NotI*, and the obtained fragment was inserted into APC/pEGFP-C1 cleaved with the same enzymes.

- 5 (c) ΔAPC-GFP: the expression vector for this protein has already been reported in the inventors' publications.

- (d) nAPC-GFP: a vector expressing only the N-terminal region of APC (comprising the heptad domain and armadillo domain). A gene encoding
10 859 amino acids of the APC N-terminus was produced by PCR using pGFP-C(*NheI*)/APC(1-8490) as the template, and the following primers:

CGACGCGTATGGCTGCTGCTTCGTATGATCAGT (SEQ ID NO: 4), and
CGACGCGTACCTGCTGTTCTTTCCCTGTC (SEQ ID NO: 5).

- 15 This PCR product was cleaved with *MluI*, and inserted into the *MluI* site of pGFP(*MluI*) vector (reported by the inventors).

- (e) n2APC-GFP: a vector expressing only the coiled-coil heptad
20 domain of the APC N-terminal region. A gene that encodes 284 amino acids of the APC N-terminus was produced by PCR using pGFP-C(*NheI*)/APC(1-8490) as the template, and the following primers:

- 5' CTAGCTAGCATGGCTGCTGCTTCGTATG 3' (SEQ ID NO: 6), and
25 3' CCTGTCCCAAGTAGGTCACGATCGATC 5' (SEQ ID NO: 7).

This PCR product was cleaved with *NheI*, and inserted into the *NheI* site of pQBI25 vector.

- 30 (f) mAPC-GFP: a vector expressing only the central region of APC. First, a gene encoding APC amino acids 860-1120 was produced by PCR using the following primers:

- CTAGCTAGCCTCGGCAACTACCATTCG (SEQ ID NO: 8), and
35 ATTAGAGCTCACTCTAGAC (SEQ ID NO: 9).

This PCR product was cleaved with *NheI* and *XbaI*, and then inserted into the *NheI* site of pQBI25 vector. Subsequently, this vector was cleaved with *EcoRI*, and inserted with a fragment containing the central region of APC, which was cut out from ΔCAPD-GFP with *EcoRI*.
5 Finally, this vector was cleaved with *HindIII* and *ApaI*, and inserted with a fragment cut out from ΔAPC-GFP using *HindIII* and *ApaI*.

(g) GFP-cAPC: a vector expressing only the C-terminal region of APC. The expression vector of this protein has already been reported in
10 the inventors' publication (Mimori-Kiyosue et al., J. Cell Biol., 148(3):505-18, 2000).

[Example 2] Transfer of cDNAs into A6 cells and screening

The inventors established cell lines with stable expression by
15 introducing the vectors obtained in Example 1 into A6 cells.

APC expression is extremely difficult in many mammalian culture cell lines. Although cells that conditionally express full-length APCs have been reported to date (Mori, P. J. et al., Proc. Natl. Acad. Sci. USA 93 (15): 7950-4, 1996), there are no reports on the
20 establishment of cell lines that stably express foreign full-length and truncated APCs. Therefore, whether A6 cells are appropriate for expressing APC was examined.

First, a preliminary experiment was performed, in which A6 cells were expressed with GFP-tag only using a pQBI25 vector (QBIogene)
25 and Effectene transfection reagent (Qiagen). GFP expression was confirmed by observation of a fluorescence-staining image under a fluorescence microscope. Cells expressing a distinct fluorescence signal other than that of GFP were not found in the investigation. Cells were cultured starting at 48-72 hours after pQBI25 vector
30 transfection in the presence of 0.6-0.8 mg/mL of G418 sulfate (Calbiochem), and resistant clones were selected. This confirmed that A6 cells readily form colonies of resistant clones. Furthermore, the expression vectors of Example 1 were introduced, and proliferation of cells emitting GFP fluorescence was confirmed. These experiments
35 were performed at the standard culturing conditions for A6 cells: room temperature and a carbon dioxide-free environment. These

results confirmed that this cell line is appropriate for visual screening of proteins that are expressed from GFP-fused APCs.

Each of the cDNAs presented in Example 1 was introduced into cells under conditions similar to those described above, and screening was performed by detecting GFP fluorescence under a fluorescence microscope. Cell lines showing stable expression of each construct were established. After establishing stably expressing cell lines, the status of mutant APC expression could be monitored easily by GFP fluorescence without further manipulating the cells, and cellular localization of mutant APC in viable cells could be detected.

[Example 3] Morphological observation of cells expressing GFP-fused full-length and truncated APCs by phase contrast microscopy

The morphology of cells expressing GFP-fused full-length and truncated APCs obtained in Example 2 was observed by phase contrast microscopy, and compared to that of the parental A6 cells. Cells were widely spread on a dish at 100% confluency, and cultured for five days or more with a medium exchange every day, until the cells became dense and polarized to an epithelial structure. Parental A6 cells and GFP-fAPC-expressing cells formed a flat monolayer structure having the morphology of an epithelium. However, in GFP-APC(Δ TSV), Δ cAPC-GFP, or nAPC-GFP-expressing cells, bulging regions instead of a flat layer are formed at a certain frequency (Fig. 2). Similar bulging regions were also observed in n2APC-GFP-expressing cells. However, bulging regions were not observed in all of the strains, and were restricted to the cells where the existing GFP fluorescence intensity was high, or more specifically, to cells with high n2APC-GFP expression levels. In order for the cells to form bulges by n2APC-GFP, the expression level had to be approximately ten times of that of GFP-APC(Δ TSV), Δ cAPC-GFP, or nAPC-GFP, as measured by their cellular fluorescence intensities.

[Example 4] Electron microscopic observation of GFP-fused full-length APC- and truncated APC-expressing cells

Electron microscopic observation was carried out for a detailed examination of the morphology of GFP-fused full-length and truncated

APC-expressing cells obtained in Example 2 (Fig. 3). Each of the cells was fixated in a 1% glutaraldehyde aqueous solution, prepared using a phosphate buffer. Sample preparation was carried out by routine procedures, and the cell surface structure was examined by scanning electron microscopy. In parental A6 cells and GFP-fAPC-expressing cells, the apical membrane of closely packed cells extended as a flat surface, and only protrusions of the microvillar and primary ciliary structures on the apical membrane were observed. In contrast, in GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP-expressing cells, several to several tens of cells formed protrusions, and regions that do not form flat cellular monolayers appeared.

Next, ultrathin sections of cells fixated in 1% glutaraldehyde aqueous solution were prepared by routine procedures, and longitudinal sections of the cells were observed by transmission electron microscopy (Fig. 4). Parental A6 cells formed a monolayer with a certain degree of intercellular space, whereas GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP-expressing cells had increased intercellular space and cell-substrate space, and clearly showed piling up of cells. Regardless of this, a typical intercellular adhesive structure was confirmed to be maintained. That is to say, despite the fact that tight junctions and adherens junctions, which are intercellular adhesive structures characteristic of the apical region of a cell, were observed in mutant APC-expressing cells according to electron microscope images, underneath these junctions, intercellular space and cell-substrate space were significant. On the contrary, in GFP-fAPC-expressing cells, absolutely no cell-cell and cell-substrate space were observed, and the cells formed parallel rows with the monolayer. These results showed that although APC has a function of strengthening cell-cell adhesion, mutant APCs do not have this function, and that both cell-cell and cell-matrix adhesions are weakened, probably by a dominant negative effect.

More specifically, whereas full-length APC has a function of strengthening cell-cell and cell-matrix adhesions, this function is lost in mutant APCs without the C-terminus; cells are detached from the matrix due to dominant negative effects and then become piled up.

Although the reason why n2APC-GFP requires a high expression level for cells to pile up (has low cell piling up activity) is still unknown, the following two possibilities were considered. 1) APC is reported to form a homodimer with its coiled-coil region at N-terminus (Josllyn, G., et. al., Proc. Natl. Acad. Sci. U S A. 1993; 90(23):11109-13). However, stable complex formation within cells seems to require also the armadillo repeat region since n2PAC-GFP can hardly co-localize with endogenous full-length APC while nAPC-GFP strongly co-localizes with APC. Accordingly, mutants that can form a stable complex with endogenous APC are more likely to exhibit dominant negative effects. 2) Asef, a regulatory factor of the actin skeleton, is reported to bind to the armadillo repeat region to enhance cell motility (Kawasaki, Y., et al., Science (2000) 289(5482), 1194-1197), therefore, cellular adhesion and morphology might have altered due to the synergistic effects of a dominant negative effect and enhanced cell motility by Asef.

[Example 5] Fluorescence microscopic observation of GFP-fused full-length APC- and truncated APC-expressing cells

The intercellular adhesive structures of GFP-fused full-length APC- and truncated APC-expressing cells obtained in Example 2 were immunostained using anti-ZO-1 antibody, observed by fluorescence confocal microscopy, and compared with that of the parental A6 cells (Fig. 5). ZO-1 is a component of tight junction (TJ), which is one of the intercellular adhesive units in the epithelial or endothelial cell layer. At the apical plasma membrane of cells that have normal epithelial polarity, ZO-1 is distributed in the continuous border surrounding the cell. In A6 cells and GFP-fAPC-expressing cells, ZO-1 was observed to be continuously present along the cellular border, and the cells were observed to have a normal epithelial morphology. In GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP-expressing cells, normal staining of ZO-1 was observed in regions where cells are not piled up, and even in the piled up regions, continuous strands of ZO-1 were observed. This result showed that the cells expressing GFP-APC(Δ TSV), Δ cAPC-GFP, and nAPC-GFP undergo piling-up while maintaining the intercellular adhesions (TJ, AJ).

These findings showed that mutant APCs affect cellular morphology, and inhibit the monolayering of normal epithelial cells. Since piled up cells maintain intercellular adhesions (TJ, AJ), which resembles the polyp formation phenomenon in model mice, these cells
5 can be used as a model system.

[Example 6] Cellular localization of full-length APC and mutant APCs
GFP-fAPC was localized in the basolateral membrane and junction regions of A6 cells, which have taken on the morphology of polarized
10 epithelial cells. The present inventors have reported in literature that a similar construct, fAPC-mGFP (a publicly disclosed full-length APC construct where GFP is inserted inside of the APC), is localized in the basolateral membrane of A6 cells (Mimori-Kiyosue, Y. and Tsukita, S., J. Cell Biol., 154(6):1105-1109, 2001). However,
15 localization of GFP-APC(Δ TSV), Δ cAPC-GFP, mAPC-GFP, nAPC-GFP, and n2APC-GFP in the basolateral membrane was markedly inhibited. Therefore, APC is considered to be localized in the basolateral membrane and junction regions due to TSV, its C-terminal PDZ binding motif. Since DLG, one of the PDZ proteins localized in the basolateral
20 membrane and junction regions, is reported to bind to the APC C-terminus (Matsumine, A., et al., Science. 1996;272(5264):1020-3), APC localization in the basolateral membrane and junction regions is considered to be dependent on PDZ-proteins such as DLG.

In A6 cells that have become polarized and formed cell-cell
25 adhesions, nAPC-GFP was found to be localized in the junction regions. However, this localization was different from that of full-length APC, which can localize not only to the junction regions but also to the basolateral membrane. Therefore, nAPC-GFP is considered to maintain only the binding with some of the APC binding proteins:
30 junction-localized proteins.

Furthermore, when microtubules were completely depolymerized by adding a microtubule-depolymerizing agent (nocodazole) under conditions in which cell density is low and cell-cell adhesion is not seen, GFP-fAPC that was localized on the microtubules moved to
35 the basal side of the cell membrane, distributed in a striated pattern, and co-localized with DLG (Fig. 6). However, GFP-APC(Δ TSV),

Δ APC-GFP, mAPC-GFP, nAPC-GFP, and n2APC-GFP were dispersed in the cytoplasm; DLG co-localization and the formation of a striated pattern as in GFP-fAPC was not observed. These results showed that APC binding to the basal side of the cell membrane requires the binding of PDZ proteins, such as DLG, in a manner that depends on TSV, a PDZ binding motif at the C-terminus of APC.

[Example 7] Cell spreading activity analysis of full-length APC and mutant APCs

Next, cell spreading assays were performed to examine the effects of full-length APC and mutant APCs on cell motility. Each of the cell lines was treated with trypsin, collected from dish, and plated onto cover glasses. The cover glasses were taken out for fixation 15, 30, 60, and 120 minutes later, and actin was stained with rhodamine-phalloidin to visualize cell shape. Each sample was photographed under a fluorescence microscope, area of the region of cells stained with rhodamine-phalloidin was measured, and the average area for each cell was determined and plotted (Fig. 7). As a result, cell spreading was significantly enhanced only in cells expressing fAPC-GFP. Next, to examine whether cell spreading activity is due to the stabilization of microtubules, a similar assay was performed under conditions in which the microtubules were depolymerized by nocodazole addition. As expected, significantly enhanced cell spreading was only observed in GFP-fAPC-expressing cells. These results showed that in addition to stabilizing microtubules, APC has cell spreading activity, and that the PDZ-binding function at the C-terminus is important for this activity. As indicated by the APC localization shown in Example 6, since APC localizes to the basolateral membrane by binding to PDZ proteins such as DLG via its C-terminal PDZ-binding motif, localization to the cell membrane via PDZ proteins is considered necessary for normal APC function. Mutant APCs do not have the function of binding to cell membrane and cannot express normal functions. Moreover, they are considered to exhibit dominant negative effects.

These results suggest that APC localizes to the cell membrane by binding to PDZ proteins through its C-terminus, and contributes

to the regulation of cell motility and maintenance of cell morphology.

Industrial Applicability

5 The present invention provides mutant APC proteins which can
induce piling up of cells. Cells expressing these proteins are useful
for screening compounds that inhibit piling up of cells. Furthermore,
the present inventors elucidated, for the first time, that cells
expressing mutant APCs maintain the intercellular adhesive structure,
and yet exhibit piling up of cells. Elucidation of the mechanisms
10 of polyp formation and malignant formation from these findings is
very much expected.